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Introduction

(Safety Blood Supply: Role of Pathogen reduction)

Even though the blood supply is very safe, concerns regarding transmission of transfusion-related pathogens exist. Risks are reduced by donor screening, arm-preparation techniques, first aliquant diversion, screening for pathogens and, in many countries, pathogen reduction/eradication technology. The risk for viral infection from transfusion is now reported at 1:2,135,000 for HIV, 1:138,700-233,000 for HBV, 1:1935,000 for HCV, 1:250,000 for HTLV 1 and 2 (after screening peripherally), 1:1,000,000 HAV, 1:10,000 for parvovirus B19, and 1:250 unfiltered blood CMV. Prior to NAT testing, risk for WNV is 1:833 (risk is suspected to decrease with NAT testing). Risk for bacterial infection is 1:500,000 RBC units or 1:13,500 random or apheresis platelet units. Two technologies are FDA approved for screening of platelet units, though not all bacterial contamination is thought to be eliminated by such screening. Seroprevalence of parasite *Trypanosome cruzi* 1:7,500 to 33,000, *Babesia microti* up to 1:100 levels vary depending on donor population. Also, risks are unknown for Leishmaniasis and Toxoplasma. Other viruses known to be transmitted by transfusion but with less or unknown clinical significance include: Transfusion-Transmitted Virus (TTV), a non-enveloped single strand DNA virus, SEN –V (a non-enveloped single strand DNA virus) which is involved in 83% of non A-E Hepatitis, and Hepatitis G virus (single strand RNA enveloped virus). Wide prevalence has been demonstrated in donor populations. Though the risk is low, significant risk for transfusion-transmitted infections remains. Emerging pathogens and unknown future pathogens are not screened for or may contaminate blood components. A new tier of

protection—pathogen reduction technology—is being developed to further decrease risks. Various additives are added to blood products to inactivate viruses, bacteria, fungi, protozoa, and other known/unknown transfusion-transmitted pathogens.

(FDA perspective)

The ideal pathogen reduction would inactivate all pathogens. The procedure/process employed would not damage the transfusion product. Any additives used would preferably be safe to administer to all patients. Many causes are present to explain why there is a decrease in pathogen reduction efficacy. In donor blood, viral loads or titer may be large. Resistant forms of the pathogen can be present in the components processed. Secondary to the geometry of a collection bag, the additive may be inaccessible to the pathogen. The pathogen reduction reaction may not be optimal secondary to poor light delivery (re: geometry) or an interfering substance may not be present (bind to pathogen reduction additive, absorb light, quench reaction). With any process involving skilled labor, human error can be present.

The pathogen reduction process may cause damage to the components, resulting in decreased retention of red cells and platelets or a decrease in coagulation proteins in FFP. After passing through Phase III trials, toxicity may not be revealed until large scaled exposure is seen.

Early in evaluating pathogen reduction methods models for *in vitro* testing of virus, bacterial, and parasites need to be performed. Ideally, at least 6-10 $\log_{10}$ reduction in pathogen

load is required to accept benefit of methodology. Peak viral load before immune response is initiated can be as high as 10^8-10^{10} units per ml.

Often times, demonstrating clinical efficiency is not feasible. Too large a study population is required to see a statistical or clinical difference. Since the initiation of NAT assays, even larger cohorts are required. Also, not all products given to patients have pathogen reduction technologies applied. Epidemiologically, multiple transmission routes/vectors are present to obtain infections.

Pathogen reduction chemicals have multiple sites of interactions. Cross-linking of lipids in cellular membranes and organelles can occur. Interacting with proteins can cause cellular signal transduction mistakes, respiratory pathway deficiencies, or structural abnormalities. Binding to nucleic acids or intercalating nucleic acid chains can lead to error in transcription, translation, or replication.

In evaluating pathogen reduction procedures, eliminating compounds clearly damaging bile blood components is necessary. Gross hemolysis (>19%), platelet dysfunction, and a decreased coagulation factor activity (>30% reduction) are benchmarks to be avoided. Subtle damage is more difficult to assess. Radio-labeling cells in autologous transfusions and monitoring survival or recovery is a method to evaluate clinical efficacy. Clinically, bleeding time may be used to assess platelet function and DLO_2 to assess red cell function.

Toxicity is also a risk with pathogen reduction. When compounds interact with nucleic acids, mutagenicity can be an issue. If present, mutagenic compounds may lead to genotoxicity, carcinogenicity, or toxicity to the reproductive system. Compounds interacting with membrane

lipids or protein can adversely affect cellular function. If such reactions are occurring, the processing must ensure the compounds are not present in the final product.

The benefits of pathogen-reduction technologies continue to decrease as techniques in donor selection, collection processes, screening test methodologies, and detection limits are improved. The risks to the recipients improve as pathogen reduction increases with less toxic and more efficient techniques developed. More benefit is apparent when new pathogens emerge and are untestable (SARS, Ebola). The likelihood of pathogen-reduction technologies replacing pathogen screening tests is not present. Such technologies may only compliment existing protocols.

Multiple pathogen-reduction technologies are reviewed below. Worldwide travel and changing demographics could bring new pathogens into focus and renew more intense research and the importance of PR technologies.

Solvent Detergents

History

A general overview of the history of solvent detergent (SD) use worldwide and in the United States is given below. SD viral inactivation technologies were first licensed for treatment of clotting factor concentrates in 1985. Prior to 1984, a combination of ethyl ether (20%) and Tween 80 (1%) were found effective in inactivating HBV and NANBHV in Factor VIII and IX concentrates with minimal loss in activity. In 1986, tri-(n-butyl) phosphate (TIOBP) and 0.2% Na cholate were shown effective in inactivating HBV, NANBHU, and HIV. From 1987 to 1988,

solvent detergent technology is nascent. Use was shown to have 6$\log_{10}$ viral load reduction in spiked samples. Coagulation factor profile was similar to untreated fresh frozen plasma (FFP). Removal of additives was 99.97% efficient. During the period from 1991-1995, approximately $2x10^6$ units of FFP were transfused without significant adverse sequelae. SD FFP has been used in Germany and Switzerland since the early 1990s and is currently used in Austria, Belgium, France, the Netherlands, and Norway, as well. Initial tests from 1990-1992 with TNBP (1% organic solvent) and Triton +100 (1% non-ionic detergent) for 4 hours at 30θC had >6 $\log_{10}$ HBV, >5 $\log_{10}$ HCV, and >6 $\log_{10}$ HIV reduction. In France, 1% TNBP and 1% octoxynol9 were evaluated and had similar coagulation factor results of untreated FFP. In some countries in Europe, SD FFP completely replaced conventional FFP. No systemic mutagenic or reproductive mal-effects have been noted. In the US, the FDA required proof of efficacy and safety. Between 1996 and 1998, SD procedures were found to be ineffective against non-enveloped viruses. Double inventories were kept secondary to possible toxicities to neonates. Studies for neonates, infants, and pregnant women are yet to be extensive. In the US, use of SD plasma encompassed only 15% of the hospital market because of the inability to recover development costs. A case series reports thrombosis or hemorrhage in liver transplant patients. Cost, risks, and administrative problems caused further declines in use in the US. There were fewer risks noted in Europe, and frequent use there continues. SD technologies are the most widely used virucidal methods in the world. Prior to 1998, $35x10^6$ doses of SD-treated products had been given. Even though solvent detergent plasma continues to be licensed in the US, the last lot was made in 2001, and it is no longer available.

<u>Mechanism of action</u>

SD treatment disrupts lipid-containing structures of enveloped virus, bacteria, and eukaryotes. Via this action, infectivity is lost. Because the chemical reaction is non-selective, the agents must be removed before the final product is transfused. Many combinations of tri-(n-butyl) phosphate (TNBP) or ethyl ether (organic solvents) with Tween 80, sodium cholate, Triton X-100 (non-ionic detergents) have been used in the past. The combination most often used now is TNBP with Triton X-100.

Molecular biology

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The molecular structure of tri-(n-butyl) phosphate is as shown above. This compound acts as an organic solvent and extracts lipids during SD processing by extracting and sequestering into a separate micellular phase.

CH3 etc

The molecular structure of polyoxyethelene-pt-octylphenol (Triton X-100) is given above. This non-ionic detergent disrupts lipid bi-layers for easier extraction into and stabilizes TNBP.

Uses of solvent detergent-treated products from 1985 to 1997 have been wide and varied. No transmission or enveloped viruses or toxicities have been reported prior to 1997. Product use for this protocol include Factor VIII concentrates, Factor IV concentrates, prothrombin complex (proconverton, Stuart factor, b factor), Factor VII-VIIa, fibrinogen, Protein C, Factor XI, antithrombin II, thrombin fibrin glue, IMIG, IVIG, anti-D IgG, HBV-Ig, CMV-Ig, RSV-Ig, anti-tetanus, monoclonal antibody (anti-cancer) complex, partially activated prothrombin, and plasma. "Double virus elimination" procedures have been developed to decrease the risk of transmission of HAV. Such procedures involve use of monoclonal antibody affinity, chromatography, nano-filtration, heat or ultraviolet light in combination with solvent detergent.

Procedure in utilizing

In using SD technology, many steps are present in the manufacture of labile blood products (SD-FFP/ Octaplas/ Plas + SD). Up to 2500 donors per batch (about 380 liters) are processed simultaneously. The FFP is quick thawed and passed through a 1 μm filter. This filtration and an additional one serves to remove cells, cell fragments, and membrane-associated viruses, assuring aggregate-free solutions. The plasma is then treated with 1% TNBP and 1% Triton X-100/polysorbate 80/octaneynol for 4 hours at 30θC. A castor oil extraction and phase separation are performed to remove TNBP. A clean filtration is done next. The Triton X-100 is removed by hydrophobic interaction chromatography. An additional sterile filtration at 0.2 μm is executed. The treated FFP is aseptically filled in 200 ml aliquots into bags, sealed, fast frozen to δ-60θC, and stored at -30θC. QC is finished, and the batch is released. No impairment of viral inactivation is seen with protein concentrations to 90 mg/ml or lipid levels of 1,064 mg/dl triglyceride and 243 mg/dl cholesterol.

In preparation of plasma-derived products, two additional steps are performed. The first is that a Cohn-Oncley fractionation procedure is rendered on the recovered plasma bench. Fractionation is not good at adequate pathogen reduction if it is the sole method of pathogen reduction. Recipient of IVIG products in 1990's may attest to; however, it does significantly decrease viral particle titers. Secondly, pH 4 treatment at 21θC before the final filtration (decreases aggregation and inactivates viruses enveloped and non-enveloped by $2\log_{10}$).

Post-residual concentration of TNBP and Triton X-100 in the final product of Vitek product and Octapharma; TNBP was undetectable in 73% of 130 batches with 27% having 0.5-1.7 μg/ml. Triton X-100 was undetectable in 91% of 130 batches with 9% having 1.0-1.6 μg/ml.

The above were evaluated in Octaplas. A study with the Vitek product revealed less than 2 ppm TNBP quantities with one lot of 34 having 2.1 ppm. Triton X-100 has δ1 ppm with two lots of 34 having 1-2 ppm.

Costs

Producing SD FFP involves filtering, tracking, extracting, monitoring residual pathogen or chemical agents, and increasing the cost of production to 3-4 times that of untreated FFP. Previous cost effective analyses have suggested SD FFP costs go up to US $9,743,000 per quality life year gained. Some have argued that, taking into account the decreased incidence of TRALI with SD FFP, the cost would decrease to US $40,855-139,465 per life year saved. In other aspects of medicine, an accepted procedure typically costs less than $30,000 per QALY gained, *i.e.*: cholesterol reduction ($13,300) or coronary artery bypass ($26,117). In other aspects of transfusion medicine, however, higher costing measures have typically been added to reduce transfusion-transmitted disease. Costs range from $235,000 to 2.3 million per QALY. Such procedures include autologous donations, p24 antigen assay for HIV. The costs should, therefore, not be the only detriment in rejecting pathogen reduction SD technique by the general public.

Efficacy

In the initial licensure of VIPLAS/SD viral inactivation, rates were reported as vesicular stomatitis virus (VSV) $>5.7\log_{10}$, Sindbis virus $\tau 5.8\log_{10}$, HIV $\tau 6.0\log_{10}$, Bovine viral diarrheal visus (BVDV) $>6.0\log_{10}$, Hepatitis-B virus (HBV) $\tau 6.0\log_{10}$, and Hepatitis C virus $>5.0\log_{10}$ reduction in active viruses. Reductions were evaluated in multiple blood component products in 1993. Vesicular stomatitis virus (VSV) $\tau 8.8 \log_{10}$, Sendai virus $\tau 6 \log_{10}$, duck HBV $\tau 11.0 \log_{10}$,

HIV1 $\tau 6.0\log_{10}$, HIV2 $\tau 6.0\log_{10}$, CMU $\tau 5.8\log_{10}$, HSV1 $\tau 4.0\log_{10}$, reduction in tissue culture infectious dose 50. Non-enveloped viruses are not so easily inactivated and show much lower log reductions. Hepatitis A virus had a #1.22 $\log_{10}$ reduction with an outbreak in the 1990s with a Factor VIII product. Seroconversions have also been noted to Parvovirus B19 (PVB19).

A benefit of the indiscriminate activity of SD treatment is enveloped pathogens not picked up on routine screening assays will be inactivated by this technique. Examples include HIV type O and variants of HBV. There is putative protection from getting HAV or Parvovirus B19 by quantities of antibody in the final product. Anti-HAV titers of 0.8 IU/ml and titer of PVB19 8 u/ml provide protection. Concentrations of anti-HAV are 30x the prophylactic dose and antibody concentrations of anti-PVB19 are seen in IVIG and used to treat chronic infections. Screening with NAT (nucleic acid testing) may be another way to decrease transfusion transmission (10 copies/ml can be detected).

Purity of SD FFP compared to FFP is maintained with a threshold of 0.7 U/ml for coagulation factors and 1.7 mg/ml for fibrinogen. In 34 lots tested, coagulation activity ranged from 0.83-1.08 U/ml for V, VII, X, XI, and XII with 2.67 mg/ml of fibrinogen. FDA studies reveal similar potency data.

Problems associated with SD technology

Even though SD FFP has activity >0.7 U/ml (approximately 15-20% lost for individual factors) compared with untreated FFP some feel that may require an increased number of transfusions of SD FFP to get the same clinical efficacy as standard FFP. Also present is the theoretically increased risk of non-infectious complications of TRALI as the wrong type of transfusion.

Protein S is decreased by 35-50% in SD FFP, Plasmin inhibitors are decreased by 76% and alpha 2 antiplasmin is decreased by 50%. These may lead to increased bleeding risk in patients deficient in these factors.

The fact that alpha antitrypsin is absent in SD FFP has unknown clinical significance in the acute state. Other therapies are available for patients with congenital deficiencies of this enzyme. Other studies show no difference in activity as compared with baseline values.

In 10/2000 in the US, 6 patients getting orthotopic liver transplants for various underlying causes of end-stage liver disease died of thrombotic or hemorrhagic events. Shortly after this episodic use of SD FFP (PLAS+SD), it fell into severe disfavor in the US. The last lots were made in 2001. Other studies in the UK in 1999 showed equal efficacy of SD FFP and FFP. In Germany (2001), open-heart surgeries using SD FFP and FFP were compared and demonstrated equivalent improvements of hemostasis and fibrinolysis.

Vaccinia has been shown to be relatively resistant to SD treatment. Even though this is an enveloped virus, intracellular forms exist without an envelope and are infectious. Screening donors for this vaccination is already performed.

This procedure has been shown in the past not to inactivate non-enveloped viruses. Outbreaks of HAV have occurred worldwide with Factor VIII and IX concentrates in each year between 1991 and 1998. PVB19 is more difficult to evaluate; however, infection rates parallel age-matched hemophiliac children. Antibodies in pools may protect from transmission. Nanofiltration may be used, but reduction in Factor VII and VWF is seen. If higher titers of antibodies to these agents are present in the final product, protection may be rendered. SD technology also cannot be used on cellular products since membranes disintegrate.

An additional risk with SD treatment is the toxicity of the chemical additives. Triton X-100 in animal studies has a CD_{50} of 1.2-1.8 g/kg via the oral route and CD_{50} for intraperitoneal or intravenous route of 108-150 mg/kg. The lowest toxicity was found to be 33.7 mg/kg in mice and 15.7 mg/kg in rats with a CD_{50} of 605-660 mg/kg in mice and 610-615 mg/kg in rats. No deleterious synergistic effect was demonstrated when substances were given simultaneously. No mutagenicity potential, embryo toxic, or teratogenic potential was demonstrated. A TTP patient could theoretically receive 63 1 of PLAS/SD or 2.7 mg/kg. This is well below toxicity levels. Also, with present processing techniques, there often is no detectable TNBP or Triton X-100 in the final blood product.

Benefits

Many of the benefits of SD treatment rest in the fact that they are unlikely to degrade the protein being purified. Coagulation factor loss is not clinically significant, as 80% activity is required to maintain normal hemostasis. Enveloped viruses are inactivated by this process, and there was no transmission of such viruses seen after transfusion of 17,000,000 units from 1980-1993. In the United States, 3% of patients had hives, abdominal pain, nausea, vomiting, chills, headache, wheezing, fever, and hypertension with an average recovery of 90% of clotting factors in this study population.

Present Research

Future studies are necessary to evaluate safety in use of products in neonates, infants, and pregnant women. A study to evaluate the statistical/clinical relevance in the decrease of

infection transmission risk would require hundreds of thousands in a co-host group. To be undertaken, this would require a multi-center trial over several years' time.

The FDA still has SD FFP licensed and is deemed comparable to FFP donor re-tested products as acceptable alternatives for those indications held/to be held in common between the 2 products. Both products continue not to have widespread or any use.

Alternate forms of SD FFP

Caprylate has been evaluated in the use of manufacturing blood derivatives, albumin, and IVIG. In IVIG manufacture, 20 mm (0.3%) caprylate is used in place of 0.3% TNBP/cholate (0.2%) for albumin caprylate 1% w/v and pH 4.5 are used as the solvent detergent step. Caprylate (octanoate) is a white crystal insoluble in water and is a monoester of glycerine and caprylic acid. The molecular structure is

$$CH_3(CH_2)_6C)CH_2CHCH_2OH$$

This compound has been used in cosmetics since 1998 for skin conditioning as a emollient and surfactant. It has also been used to dissolve gallstones since 1989. It functions by disrupting lipid bilayer and membrane associated proteins of viruses. Increase temperature increase inactivation rate and low pH increase nonionized form.

In the last 50 years has been used in manufacture of 5% albumin as stabilizing agent, without toxic effect of final concentration of 4mm. It has been shown to have $\log_{10}$ reducing power of 4.4 for BVDV (Hepatitis C model), 4.7 for HIV, 4.2 Pseudorabies virus (PRV) in IVIG

and ≈7.1 for Sindbis, ≥ 4.7 for BVDV, ≥for PRV and ≥5.4 for HIV in albumin preparation. This Process was equivalent in viral reduction as SD treated products, however, 10% loss noted in Ig levels of IVIG. Benefit of this procedure is that caprylate already in process in manufacture of albumin and no additional steps required and minimal toxicity decreased need for removal in final product.

Another product is Uniplas (a solvent detergent treated universal plasma) where in anti-A and anti-B iso-agglutinin antibodies have been removed. Immune complex of α-A and α-B antibodies with soluble A and B antigens are hydrophobic and removed by SD treatment. Total protein and coagulation factors are similar to SD-FFP. No patients in study converted to HIV, HTLV, HBC, CMV, HAV or Parvovirus B19 to seropositivity. Two studies showed equivalent efficacy of Uniplas to Octiplas in open heart surgeries without restricting FFP to a particular blood group. Further studies recommended, to see if inactivation better than standard SD-FFP and maintain therapeutic value of the product in more clinical situations.

Phenothiazinium Dyes

Methylene Blue

Methylene Blue was first synthesized by Caro in 1876, and in 1883 Bernsthen made parent ring system, and currently this compound has been administered orally as an antiseptic, disinfectant, and an antidote for nitrate poisoning. MB use has been in treatments of methehemoglobinemia and for validation with compliance of medicines. Long use in humans ensures its safety. In 1891 MB was used to cure Malaria in 2 patients. In 1928, it was demonstrated to inactivate HSV, vaccinia in the presence of light. Nucleic acids were found to be the target of reaction in 1956 and oxygen was found to be necessary in this reaction in photodynamic activity.

Ideally, a photochemical dye first needs to penetrate membranes whether pathogen or plasma membrane to inactivate extra as well as intra cellular pathogens. Secondly, by adhering to predominately/exclusively nucleic acid compounds get little damage to red blood cells. Thirdly, need to absorb red light well (>600nm) to get decrease interference of hemoglobin absorption. Lastly need to have unbound dye not cause excessive damage to RBC or have dark toxicity.

Phenothiazine dyes are more attractive than chemical uncontrolled reactions because unwanted cell damage can be limited by absent light exposure after initial treatment completed. In the last 100 years bacterial and topical diseases (i.e. Malaria) were treated with methylene Blue. As antibiotics specific to pathogens became available in the last 50 years use has decreased. As Plasmadium falciparum has increasing incidence of chloroquine resistance and drug resistant bacteria (MRSA, VRE, Multi-resistant TB)occur in this age of HIV infection a Renaissance of interest has developed.

Mechanism of Action

Methylene Blue binds DNA in 2 ways depending on ionic strength and concentration of Mg+. MB may have external binding or internal (intercalative). MB causes guanine specific cleavage. In anoxic solution a direct electron transfer is probably responsible for strand breakage, via direct cleavage of phosphodiester bonds. In oxygen rich solution reactive oxygen species (iO2, OH, superoxides are generated. MB works with peak absorption (620-670nm) to produce a type I reaction (redox) or Type II (photodynamic, photooxidative interaction. MB also binds to viral core proteins and has been shown to cause damage to bacteria (*Proteus mirabilis*) and RBC. Bacteria and hemoglobin can reduce MB and convert to a leukomethylene form

which is neither a photosensitive nor an intercalater (max abs 340nm). MB technology has been shown to inactivate envelope and some nonenveloped viruses; however given its hydrophobic nature at concentrations of 5mm this compound cannot penetrate plasma membranes and/or intracellular viruses.

Molecular biology

The formal chemical name of Methylene blue is 3,7 bis (dimethylamino) – phenothiazine – 5 - ium chloride and classed as a thiazine dye.

The structural formula is:

Leukobase inactive form:

The lipophilicity of MB (=$\log_{10}$ partition coefficient between phosphate buffer and 1-octanol) in log P-0.10 Revealing a hydrophobic compound.

Preference of binding of MB to various organic compounds show increased binding to DNA and negative charged lipids. Albumin electrically neutral lipid had <3% bond with DNA and negatively charged lipids having 65% and 20% binding. Poly G in the same solution accounted for the remaining 15% binding in that study.

Present use in Transfusion

FFP methylene blue treated plasma has been in routine use since 1992, after research in Walter Reed in 1955. A few countries in Europe use this decontamination method. From 1991 to 2000, 2 million units have been transfused with no untoward events. MB has been used as a market in surgery to reduce methemoglobin, 50 mg TID for ifosfamide-induced encephalopathy, or 2 mg/kg in septic shock. Though MB has been used in much higher concentrations, Paul Ehrlich institution has refused to license MB-treated plasma due to toxicity concerns. Adverse health events associated with MB include a burning sensation in the mouth, nausea, vomiting, diarrhea, and gastritis. Large doses may cause abdominal and chest pain, headache, profuse sweating, mental confusion, painful urination and methemoglobinemia (MSDS). A present are two proprietary methods for processing FFP with methylene blue (Pathonact MBC Baxter and Maestronic [Mccapharma])

Procedure

In general the requirements of this product in use are 1 μm concentration MB with exposure to red light (600-700 nm) with fluence rate of 10 mW/cm^2 for 600 sec. Freeze and thawing of unit may liberate intracellular organisms. In a procedure, 3 units at a time were processed by passing the contents of a unit through a 0.65 μm membrane filtration. After filtration, a dry tab of MB (80 mg) was placed in the line. The MB dissolved during filtration. Approximately 180 J/cm^2 in less than 20 mm was applied with 590 mm peak O of light. The concentration of 1 μm MB in the unit was computer controlled. Other procedure process with 5

μm concentration with a fluence of 11.3 J/cm^2. The product is stored at -30θC. Quality control and release were performed. When needed it is then thawed and transfused as untreated FFP.

[MB cost/extra cost processing/monitoring]

In 1996 US dollars, the cost effectiveness of $111,000 to 376,650 for patients under 40 or low volume transfusions. The study showed high costs per life year gained, even in more favorable group.

Efficacy

The powers of MB reduction are increased in the presence of O_2. This procedure has demonstrated ability to inactivate enveloped viruses both DNA and RNA (give examples). Some non-enveloped viruses with large capsid pores are inactivated (i.e., WNV 5.7 $\log_{10}$ reduction, HIV 6.32 $\log_{10}$, VSV 6 log v). However, non-encapsulated viruses with tightly interdigitated capsid proteins (encephalomyocarditis virus) are not inactivated. Little bacterial inactivation is observed under viricidal phototherapeutic conditions. MB is more effective against gram + than gram – organisms. Candida Albicans and Trypanosome Brucei are also inactivated. This compound has difficulty in penetrating plasma membranes and as such intracellular organisms are not inactivated. The purity of red cells when used with an RBC product is decreased. After 42 days of storage 0.8% of the cells lyse. Membranes of red cells have dramatically increased ion leakage. MB binds to IgG and albumin (serum proteins) may further alter RBC membranes by attaching to membrane proteins. A significant benefit of this compound is its long history of use with minimal toxicity. There are however a few toxicities and side effects when used in large doses.

Problems

Over the past decade or so, MB has been used in FFP. Multiple difficulties have arisen even though at least two retrospective studies have shown no toxicity with MB PRFFP others have shown an increased incidence of TRALI. In UK/Europe no episodes of TRALI before 2001 after 1,000,000 units were transfused. Secondary to binding to proteins 10 to 30 % of coagulation factors and 20 to 24 % of fibrinogen are inactivated. The loss of activity is thought to be secondary to oxidation of histidine residues and other amino acids. Also, MB binds to alpha subunits of fibrinogen; lower binding to platelets receptors results.

Studies are present that MB does not inactivate WBC. Not only are intracellular pathogens inactivated, but neither are the white blood cells. GVHD is thus not lessened after labile blood products are treated with MB.

Up to one half of the MB added to RBC's is bound to membrane proteins or located to RBC's. The compound within the RBC's is reduced to the leukoform and can neither intercalate nor photosensitize. Binding to RBC surface proteins increases ion permeability. MB also inactivates glutathione reductase, decreases red cells ability to handle oxidation toxicity.

Toxicity in animal studies reveal LD_{50} oral route as being 1180 mg/kg and intraperitoneally 180 mg/kg for rats. In my studies oral LD_{50} was 3500mg/kg. This demonstrates a low toxicity for this compound.

Dimethyl Methylene Blue

Historically DMMB does not have as extensive a use in medicine as MB does. This compound however has some characteristics that make it more desirable than MB. When a photon is absorbed by this dye, in the presence of molecular oxygen a singlet oxygen molecule forms ¾ of the time. The reactive oxygen species often formed is a hydroxyl radical. DMMB

has 10 time more affinity for nucleic acids than does MB. A higher rate of singlet O2 formation is present with a lower redox potential. At O max of 648 nm relative yield of reactive oxygen species to MB is 1.21. DMMB has increased lipophilicity over MB (log P = +1.01 DMMB, log P = - 0.10 for MB). DMMB is better able to penetrate plasma membranes, better able to inactivate intracellular pathogens and WBC. Fewer graphs versus host disease would occur. WBC inactivation is either from inactivation of mitochondrial DNA, nuclear DNA, or both. IN the presence of DMMB and light treatment DNA does degrade and little annexin V binding phasphatidylserine is seen (a sign of apoptosis).

Molecular structure

DMMB quinicrine

Spectra of absorbance 515 Photochemistry and photobiology 2002 76(5)

At present, not able to find this compound in use of routine pathogen reduction in labile blood products.

In research studies DMMB is added to a RBC sample of 30-45% hematocrit (leukoreduced) and oxygenated, at concentration of 0.1, 1, 3.6, 4, or 10 Πm. Fluence involves a light source of cool white fluorescent bulbs or 670nm peak LED for a total of 5.4 mW/cm^2 for 2

seconds to 15 minutes. Some studies add citrate to decrease the osmolarity lysis and quinicrine to decrease lysis secondary to membrane binding.

Efficacy reduction power, pathogen types, potency, safety and purity

Many viral models have been studied to evaluate the pathogen reduction power of DMMB. Duck HBV (human hepatitis B virus model) was reduced 5.77 $\log_{10}$, VSV reduced 3.54 -4.4 $\log_{10}$ with quinicrine added the reduction power is increased 3x exponentially to 7 $\log_{10}$. BVDV is reduced greater than 4.7 $\log_{10}$. Bacteriophage I 6 is reduced 5.6 $\log_{10}$ and R17 is reduced >7 $\log_{10}$ (both as models of non-enveloped viruses). Intracellular VSV an PRV show similar kinetic curves as extra-cellular reactions. The greater PR ability of this compound is secondary to the > 50% singlet oxygen formation, nucleic acid binding, and plasma membrane penetration lead to a 10x increase in viral inactivation over MB. T cell inactivation also occurs at a dose 5x less than required for viral inactivation. A large safety margin is available to prevent TA- GVHD.

Problems

The safety of this product is unknown. The final product requires decontamination. Research is being performed with absorbent filtration. Dark toxicity appears greater than MB. In liquid RBC products treated with DMMB more lysis is seen. When DMMB is used as PR the degree of hemolysis is up to 25%. The presence of citrate decreases to 2%. When quinicrine is added to the mixture a competitive binding occurs at the RBC membranes and hemolysis

decreases to 1.2%. The remaining hemolysis is thought to be due to reactive oxygen species created by unbound dye. Three putative mechanisms direct the hemolysis 1) colloidal osmotic hemolysis from cell membrane ion leaks, 2) cell membrane damage from membrane bound dye, 3) damage to membrane from photosensitizer generating photodamage. Additional / different agents are needed to decrease hemolysis to the FDA goal of <1%.

Benefits

DMMB can continue to act as a photosensitizer as hemoglobin does not appreciatively absorb above 630nm. Even though hemolysis occurs after treatment with DMMB, RBC morphology is unchanged. With protein electrophoresis the bending pattern of RBC membrane proteins remains unchanged. No changes in RBC antigens are also demonstrated. Reportedly there is little change in osmotic fragility lysis, potassium efflux, ATP or 2, 3 DPG concentrations. DMMB does not bind IgG; no new antigens are formed.

Future applications of the DYE

The best method to detect lysis is by an increase of K+ levels. Increased K+ levels is more sensitive than detecting apoptosis markers by flow cytometry. Free hemoglobin is also a less sensitive indicator of hemolysis than K+ efflux. New methods to protect against hemolysis, better than citrate /quinicrine need to be developed in the future.

Research in developing a closed system for DMMB pathogen reduction is desirable. A need to evaluate the toxicity of DMMB is present. Further research in the types of viruses, inactivation of intracellular and extracellular PR needs to be further evaluated.

Studies of other photosensitizing systems or dyes could be developed. New methylene blue (structure) has been demonstrated to have equal or greater bacterial cidal activity as DMMB with decreased toxicity to mammalian cells. NMB has bee used in stains for the laboratory for 50 years. The site of activity appears to be ribosomal based. Phenothiazinium series are not only good against viruses and bacteria but have evidence of fungal, plasmodium and trypanocidal activity. This form of PR had not been fully explored.

Iodine

Iodine is often used as a topical disinfectant and has great antimicrobial properties. In labile blood products iodine can be used in free form or bound to protein. The mechanism of action are many fold. Iodine acts with the double bonds in unsaturated fatty acids, amino groups on amino acids (i.e. histidine, arginine, and lysine), oxidation of sulfhydral groups (cystein), react with phenol groups (tyrosine) and may react with nucleotides (cytosine, guanine, adenine). Iodine is a strong oxidizer but reactions are not localized or fully controlled. Now used as a topical antiseptic during blood collection. A combination of povidine iodine with isopropyl

alcohol was found to be more effective than cetrimide/chlorhexidine with isopropyl alcohol. Also of benefit is the inexpensive nature of this PR system.

In viral reduction power: VSV >6.2 $\log_{10}$, EMCV >6.2 $\log_{10}$, SinV >6.9 $\log_{10}$, PRV >4.31 $\log_{10}$, and HIV >3.3$\log_{10}$ reduction in viral activity. At least 70% of the coagulation factors remain intact. Difficulties with this system include thyroid abnormalities, allergic reactions. The toxicity levels are determined and free formed reactions are not selective. Removal of iodine after reaction is necessary and this system not ideal for PR in cellular labile blood products.